PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/53, 1/15, A61K 7/13, 7/06, D21C 5/00 // (C12N 1/15, C12R 1:66)

A1

(11) International Publication Number: WO 95/33836

(43) International Publication Date: 14 December 1995 (14.12.95)

(21) International Application Number: PCT/US95/06815

(22) International Filing Date: 31 May 1995 (31.05.95)

(30) Priority Data:

08/253,781	3 June 1994 (03.06.94)	US
08/441,146	15 May 1995 (15.05.95)	US

(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(71) Applicants: NOVO NORDISK BIOTECH, INC. [US/US]; 1445 Drew Avenue, Davis, CA 95616-4880 (US). NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventors: BERKA, Randy, M.; 3609 Modoc Place, Davis, CA 95616 (US). BROWN, Stephen, H.; 3708 Miwok Place, Davis, CA 95616 (US). XU, Feng; 1534 Carmel Valley Drive, Woodland, CA 95776 (US). SCHNEIDER, Palle; Rydtoften 43, DK-2750 Bellerup (DK). AASLYNG, Dorrit, Anita; Gartnerkrogen 69, DK-3500 Værløse (DK). OXENBØLL, Karen, M.; Slotsvej 76, DK-2920 Charlottenlund (DK).

(74) Agents: ZELSON, Steve, T. et al.; Novo Nordisk of North America, Inc., Suite 6400, 405 Lexington Avenue, New York, NY 10174 (US).

(54) Title: PHOSPHONYLDIPEPTIDES USEFUL IN THE TREATMENT OF CARDIOVASCULAR DISEASES

(57) Abstract

The present invention relates to isolated nucleic acid constructs containing a sequence encoding a *Myceliophthora* laccase, and the laccase proteins encoded thereby.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

Phosphonyldipeptides useful in the treatment of cardiovascular diseases

Field of the Invention

The present invention relates to isolated nucleic acid fragments encoding a fungal oxidoreductase enzyme and the purified enzymes produced thereby. More particularly, the invention relates to nucleic acid fragments encoding a phenol oxidase, specifically a laccase, of a thermophilic ascomycete, *Myceliophthora*.

Background of the Invention

Laccases (benzenediol:oxygen oxidoreductases) are multi-copper-containing enzymes that catalyze the oxidation of phenolics. Laccase-mediated oxidations result in the production of aryloxy-radical intermediates from suitable phenolic substrate; the ultimate coupling of the intermediates so produced provides a combination of dimeric, oligomeric, and polymeric reaction products. Such reactions are important in nature in biosynthetic pathways which lead to the formation of melanin, alkaloids, toxins, lignins, and humic acids. Laccases are produced by a wide variety of fungi, including ascomycetes such as *Aspergillus*, *Neurospora*, and *Podospora*, the deuteromycete *Botrytis*, and basidiomycetes such as *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*, *Trametes*, and perfect forms of *Rhizoctonia*. Laccase exhibits a wide range of substrate specificity, and each different fungal laccase usually differs only quantitatively from others in its ability to oxidize phenolic substrates. Because of the substrate diversity, laccases generally have

found many potential industrial applications. Among these are lignin modification, paper strengthening, dye transfer inhibition in detergents, phenol polymerization, juice manufacture, phenol resin production, and waste water treatment.

Although the catalytic capabilities are similar, laccases made by different fungal species do have different temperature and pH optima, and these may also differ depending on the specific substrate. A number of these fungal laccases have been isolated, and the genes for several of these have been cloned. For example, Choi *et al.*(Mol. Plant-Microbe Interactions 5: 119-128, 1992) describe the molecular characterization and cloning of the gene encoding the laccase of the chestnut blight fungus, *Cryphonectria parasitica*. Kojima *et al.* (J. Biol. Chem. 265: 15224-15230, 1990; JP 2-238885) provide a description of two allelic forms of the laccase of the white-rot basidiomycete *Coriolus hirsutus*. Germann and Lerch (Experientia 41: 801,1985; PNAS USA 83: 8854-8858, 1986) have reported the cloning and partial sequencing of the *Neurospora crassa* laccase gene. Saloheimo *et al.*(J. Gen. Microbiol. 137: 1537-1544, 1985; WO 92/01046) have disclosed a structural analysis of the laccase gene from the fungus *Phlebia radiata*.

Attempts to express laccase genes in heterologous fungal systems frequently give very low yields(Kojima et al., *supra*; Saloheimo et al., Bio/Technol. 9: 987-990, 1991). For example, heterologous expression of *Phlebia radiata* laccase in *Trichoderma reesei* gave only 20 mg per liter of active enzyme(Saloheimo, 1991, *supra*). Although laccases have great commercial potential, the ability to express the enzyme in significant quantities is critical to their commercial utility. At the present time there are no laccases which are expressed at high levels in commercially

utilized hosts such as *Aspergillus*. Thus, the need exists for a laccase which can be produced in commercially useful (i.e., gram per liter or more) quantities. The present invention fulfills such a need.

Summary of the Invention

The present invention relates to a DNA construct containing a nucleic acid sequence encoding a *Myceliophthora* laccase. The invention also relates to an isolated laccase encoded by the nucleic acid sequence. Preferably, the laccase is substantially pure. By "substantially pure" is meant a laccase which is essentially (i.e.,≥90%) free of other non-laccase proteins.

In order to facilitate production of the novel laccase, the invention also provides vectors and host cells comprising the claimed nucleic acid sequence, which vectors and host cells are useful in recombinant production of the laccase. The sequence is operably linked to transcription and translation signals capable of directing expression of the laccase protein in the host cell of choice. A preferred host cell is a fungal cell, most preferably of the genus *Aspergillus*. Recombinant production of the laccase of the invention is achieved by culturing a host cell transformed or transfected with the construct of the invention, or progeny thereof, under conditions suitable for expression of the laccase protein, and recovering the laccase protein from the culture.

The laccases of the present invention are useful in a number of industrial processes in which oxidation of phenolics is required. These processes include lignin manipulation, juice manufacture, phenol polymerization and phenol resin production.

Brief Description of the Figures

Figure 1 shows a restriction map of a 7.5 *Eco*RI fragment in pRaMB1. The region hybridizing to the *N. crassa* laccase gene probe is shaded.

Figure 2 illustrates the nucleotide(SEQ ID NO: 1) and amino acid (SEQ ID NO: 2) sequence of *Myceliophthora thermophila* laccase. Lower case letters in the nucleotide sequence indicate the position of introns. Putative TATA and CAAT sequences in the promoter region are in boldface and underlined. Consensus lariat structures(PuCTPuAC)within the introns are underlined.

Figure 3 illustrates the construction of plasmid pRaMB5.

Detailed Description of the Invention

Myceliophthora thermophila is a thermophilic Ascomycete originally described by Apinis (Nova Hedwigia 5: 57-78, 1963) and named *Sporotrichum thermophile*. Subsequent taxonomic revisions have placed this organism in the genus *Chrysosporium* (Von Klopotek, A. Arch. Microbiol. 98: 365-369, 1974) and later to *Myceliophthora* (Van Oorschot, Persoonia 9: 401-408, 1977). A number of organisms known by other names also appear to belong to this species. These include *Sporotrichum cellulophilum* (U.S. Patent No. 4,106,989); *Thielavia thermophila* (Fergus and Sinden, Can. J. Botany 47: 1635-1637, 1968); *Chrysosporium fergussi* and *Corynascus thermophilus* (Von Klopotek, *supra*). This species is known as a source of a number of different industrially useful enzymes, such as cellulases, ß-glucosidase and xylanase (see, e.g., Oberson et al., Enzyme Microb. Technol. 14 : 303-312, 1992; Merchant et al., Biotechnol. Lett. 10: 513-516, 1988; Breuil et al. Biotechnol. Lett. 8: 673-676, 1986; Gilbert et al., Bioresource Technol. 39: 147-154, 1992). It has now been determined that *Myceliophthora* produces a neutral pH

laccase, and the gene encoding this laccase can be used to produce large yields of the enzyme in convenient host systems such as *Aspergillus*.

To identify the presence of a laccase gene in *Myceliophthora*, a 5' portion of the *Neurospora crassa* laccase gene(*lcc1*) is used as a probe, under conditions of mild stringency, in southern hybridization of total genomic DNA of different fungal species. An approximately 12 kb laccase specific sequence is detected in the *Myceliophthora* DNA. The *N. crassa* fragment is then used to screen about 20,000 plaques of an *M. thermophila* genomic DNA library in a λ EMBL4 bacteriophage cloning vector. Eight plaques strongly hybridize with the probe; from these eight, DNA is isolated from three. Each of these clones contains a 7.5 *Eco*RI fragment which also hybridizes to the probe (Figure 1). One of the fragments is subcloned into pBR322 to generate plasmid pRaMB1. Using the *lcc1* probe, the position of the coding region of the clone is determined. The entire *M. thermophila* coding region appears to be contained with a 3.2 kb *Nhe*I-*Bgl*II segment, which is then cloned into pUC119 and sequenced by the primer walking method.

Once the sequence is determined, the positions of introns and exons within the gene is assigned based on alignment of the deduced amino acid sequence to the corresponding *N. crassa* laccase gene product. From this comparison, it appears that the gene (*lccM*) of *M. thermophila* is composed of seven exons(246, 79, 12, 70, 973, 69 and 411 nucleotides) interrupted by six introns (85, 84, 102, 72, 147, and 93 nucleotides). The coding region, excluding intervening sequences, is very GC-rich(65.5% G+C) and encodes a preproenzyme of 620 amino acids: a 22 amino acid signal peptide, a 25 amino acid propeptide, and a mature laccase comprising 573 amino acids. The sequence of

the *M. thermophila* gene and the predicted amino acid sequence is shown in Figure 2 (SEQ ID NOS: 1 and 2).

The laccase gene is then used to create an expression vector for transformation of *Aspergillus* host cells. The vector, pRaMB5 contains the *A. oryzae* TAKA-amylase promoter and terminator regions. The construction of pRaMB5 is outlined in Figure 3. *Aspergillus* cells are cotransformed with the expression vector and a plasmid containing the *pyrG* or *amdS* selectable marker. Transformants are selected on the appropriate selective medium containing ABTS. Laccase-producing colonies exhibit a green halo and are readily isolatable. Selected transformants are grown up in shake flasks and culture broths tested for laccase activity by the syringaldazine method. Shake flask cultures are capable of producing 0.2 or more g/liter of laccase, and in fermentors, yields of over 1-2 g/liter are observed.

According to the invention, a *Myceliophthora* gene encoding a laccase can be obtained by methods described above, or any alternative methods known in the art, using the information provided herein. The gene can be expressed, in active form, using an expression vector. A useful expression vector contains an element that permits stable integration of the vector into the host cell genome or autonomous replication of the vector in a host cell independent of the genome of the host cell, and preferably one or more phenotypic markers which permit easy selection of transformed host cells. The expression vector may also include control sequences encoding a promoter, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes. To permit the secretion of the expressed protein, nucleotides encoding a signal sequence may be inserted prior to the coding sequence of the gene. For expression under the direction of control sequences, a laccase gene to be used

according to the invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can direct the transcription of the laccase gene, include but are not limited to the prokaryotic ß-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; and in Sambrook et al., Molecular Cloning, 1989.

The expression vector carrying the DNA construct of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will typically depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the laccase DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA construct of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis*

α-amylase gene (*amy*L), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amy*M), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amy*Q), or the promoters of the *Bacillus subtilis* xylA and xylB genes. In a yeast host, a useful promoter is the eno-1 promoter. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* or *A. awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred are the TAKA-amylase and *glaA* promoters.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the laccase of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter. The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B.subtilis* or *B.licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Examples of *Aspergillus* selection markers include *amdS*, *pyrG*, *argB*, *niaD*, *sC*, and *hygB*, a marker giving rise to hygromycin resistance. Preferred for use in

an *Aspergillus* host cell are the *amdS* and *pyrG* markers of *A. nidulans* or *A. oryzae*. A frequently used mammalian marker is the dihydrofolate reductase (DHFR) gene. Furthermore, selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

It is generally preferred that the expression gives rise to a product which is extracellular. The laccases of the present invention may thus comprise a preregion permitting secretion of the expressed protein into the culture medium. If desirable, this preregion may be native to the laccase of the invention or substituted with a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions. For example, the preregion may be derived from a glucoamylase or an amylase gene from an *Aspergillus* species, an amylase gene from a *Bacillus* species, a lipase or proteinase gene from *Rhizomucor miehei*, the gene for the α-factor from *Saccharomyces cerevisiae* or the calf preprochymosin gene. Particularly preferred, when the host is a fungal cell, is the preregion for *A. oryzae* TAKA amylase, *A. niger* neutral amylase, the maltogenic amylase form *Bacillus* NCIB 11837, *B. stearothermophilus* α-amylase, or *Bacillus licheniformis* subtilisin. An effective signal sequence is the *A. oryzae* TAKA amylase signal, the *Rhizomucor miehei* aspartic proteinase signal and the *Rhizomucor miehei* lipase signal.

The procedures used to ligate the DNA construct of the invention, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al.. Molecular Cloning, 1989).

The cell of the invention either comprising a DNA construct or an expression vector of the invention as defined above is advantageously used as a host cell in the recombinant production of a enzyme of the invention. The cell may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The host cell may be selected from prokaryotic cells, such as bacterial cells. Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E.coli*. The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The host cell may also be a eukaryote, such as mammalian cells, insect cells, plant cells or preferably fungal cells, including yeast and filamentous fungi. For example, useful mammalian cells include CHO or COS cells. A yeast host cell may be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces*

cerevisiae. Useful filamentous fungi may selected from a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Alternatively, a strain of a *Fusarium* species, e.g. *F. oxysporum*, can be used as a host cell. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023. A suitable method of transforming *Fusarium* species is described by Malardier et al., 1989.

The present invention thus provides a method of producing a recombinant laccase of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the enzyme and recovering the enzyme from the cells and/or culture medium. The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the laccase of the invention. Suitable media are available from commercial suppliers or may be prepared according to published formulae (e.g. in catalogues of the American Type Culture Collection).

In a preferred embodiment, the recombinant production of laccase in culture is achieved in the presence of an excess amount of copper. Although trace metals added to the culture medium typically contain a small amount of copper, experiments conducted in connection with the present invention show that addition of a copper supplement to the medium can increase the yield of active enzyme many-fold. Preferably, the copper is added to the medium in soluble form, preferably in the form of a soluble copper salt, such as copper chloride, copper sulfate, or copper acetate. The final concentration of copper in the medium should be in the

range of from 0.2-2mM, and preferably in the range of from 0.05-0.5mM. This method can be used in enhancing the yield of any recombinantly produced fungal laccase, as well as other copper-containing enzymes, in particular oxidoreductases.

The resulting enzyme may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like. Preferably, the isolated protein is about 90% pure as determined by SDS-PAGE, purity being most important in food, juice or detergent applications.

In a particularly preferred embodiment, the expression of laccase is achieved in a fungal host cell, such as *Aspergillus*. As described in detail in the following examples, the laccase gene is ligated into a plasmid containing the *Aspergillus oryzae* TAKA α-amylase promoter, and the *Aspergillus nidulans amdS* selectable marker. Alternatively, the *amdS* may be on a separate plasmid and used in co-transformation. The plasmid (or plasmids) is used to transform an *Aspergillus* species host cell, such as *A. oryzae* or *A. niger* in accordance with methods described in Yelton et al. (PNAS USA 81: 1470-1474,1984).

Those skilled in the art will recognize that the invention is not limited to use of the nucleic acid fragments specifically disclosed herein, for example, in Figure 1. It will also be apparent that the invention encompasses those nucleotide sequences that encode the same amino acid sequences as depicted in Figure 1, but which differ from the specifically depicted nucleotide sequences

by virtue of the degeneracy of the genetic code. Also, reference to Figure 1 in the specification and the claims will be understood to encompass both the genomic sequence depicted therein as well as the corresponding cDNA and RNA sequences, and the phrases "DNA construct" and "nucleic acid sequences" as used herein will be understood to encompass all such variations. "DNA construct" shall generally be understood to mean a DNA molecule, either single- or double-stranded, which may be isolated in partial form from a naturally occurring gene or which has been modified to contain segments of DNA which are combined and juxtaposed in a manner which would not otherwise exist in nature.

The *Myceliophthora* laccase described herein has a particularly high specific activity on a syringaldazine substrate relative to other known ascomycete or deuteromycete extracellular laccases in which such specific activity has been described. The present sequence provides a means by which other such ascomycete and/or deuteromycete laccases can also be isolated. Identification and isolation of laccase genes from sources other than those specifically exemplified herein can be achieved by utilization of the methodology described in the present examples, with publicly available ascomycete and deuteromycete strains. In particular, the specific sequence disclosed herein can be used to design primers and/or probes useful in isolating similar laccase genes by standard PCR or southern hybridization techniques. The present invention thus encompasses those ascomycete and deuteromycete laccases which have a specific activity of at least about 30 SOU/mg, and preferably at least about 40 SOU/mg, "SOU" being defined as μmole of substrate oxidized per minute as measured with syringaldazine as a substrate, at optimum pH.

In addition, the invention also encompasses other *Myceliophthora* laccases, including alternate forms of

laccase which may be found in *M. thermophila* and as well as laccases which may be found in other fungi falling within the definition of *Myceliophthora* as defined by Van Oorschot, 1977, *supra*. Identification and isolation of laccase genes from sources other than those specifically exemplified herein can be achieved by utilization of the methodology described in the preseent examples, with publicly available *Myceliophthora* strains. Alternately, the sequence disclosed herein can be used to design primers and/or probes useful in isolating laccase genes by standard PCR or southern hybridization techniques. Other named *Myceliophthora* species include *Myceliphthora hinnulea* (Awao et al., Mycotaxon. 16: 436-440, 1983), *Myceliophthora vellerea* (Guarro et al, Mycotaxon. 23: 419-427, 1985), and *Myceliophthora lutea* Costatin. Also encompassed are laccases which are synonyms, e.g., anamorphs or perfect states of species or strains of the genus *Myceliophthora*. Strains of *Myceliophthora* are readily accessible to the public in a number of culture collections, such as ATCC 48102, 48103, 48104 et al.; CBS 117.65, 131.65, 379.65 et al., DSM 1799 (*M. thermophila*), ATCC 52474, CBS 539.82, 540.82 et al. (*M. hinnulea*), DSM 62114, CBS 146.50, 147.50, 157.51 et al (*M. lutea*), and CBS 478.76, 479.76 and 715.84(*M. vellerea*). The invention also encompasses any variant nucleotide sequence, and the protein encoded thereby, which protein retains at least about an 80%, preferably at least 85%, and most preferably at least 90-95% homology with the amino acid sequence depicted in Figure 1, and which qualitatively retains the laccase activity of the sequence described herein. Useful variants within the categories defined above include, for example, ones in which conservative amino acid substitutions have been made, which substitutions do not significantly affect the activity of the protein. By conservative substitution is meant that amino acids of the same class may be substituted

by any other of that class. For example, the nonpolar aliphatic residues Ala, Val, Leu, and Ile may be interchanged, as may be the basic residues Lys and Arg, or the acidic residues Asp and Glu. Similarly, Ser and Thr are conservative substitutions for each other, as are Asn and Gln. It will be apparent to the skilled artisan that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active enzyme. Retention of the desired activity can readily be determined by conducting a standard ABTS oxidation method, such as is described in the present examples.

The protein can be used in number of different industrial processes. These processes include polymerization of lignin, both Kraft and lignosulfates, in solution, in order to produce a lignin with a higher molecular weight. A neutral/alkaline laccase is a particular advantage in that Kraft lignin is more soluble at higher pHs. Such methods are described in, for example, Jin et al., Holzforschung 45(6): 467-468, 1991; US Patent No. 4,432,921; EP 0 275 544; PCT/DK93/00217, 1992.

The laccase of the present invention can also be used for in-situ depolymerization of lignin in Kraft pulp, thereby producing a pulp with lower lignin content. This use of laccase is an improvement over the current use of chlorine for depolymerization of lignin, which leads to the production of chlorinated aromatic compounds, which are an environmentally undesirable by-product of paper mills. Such uses are described in, for example, Current opinion in Biotechnology 3: 261-266, 1992; J. Biotechnol. 25: 333-339, 1992; Hiroi et al., Svensk papperstidning 5: 162-166, 1976. Since the environment in a paper mill is typically alkaline, the present laccase is more useful for this purpose than other known laccases, which function best under acidic conditions.

Oxidation of dyes or dye precursors and other chromophoric compounds leads to decolorization of the compounds. Laccase can be used for this purpose, which can be particularly advantageous in a situation in which a dye transfer between fabrics is undesirable, e.g., in the textile industry and in the detergent industry. Methods for dye transfer inhibition and dye oxidation can be found in WO 92/01406; WO 92/18683; EP 0495836; Calvo, Mededelingen van de Faculteit Landbouw-wetenschappen/Rijiksuniversitet Gent.56: 1565-1567, 1991; Tsujino et al., J. Soc. Chem.42: 273-282, 1991.

The laccase is particularly well-suited for use in hair dyeing. In such an application, the laccase is contacted with a dye precursor, preferably on the hair, whereby a controlled oxidation of the dye precursor is achieved to convert the precursor to a dye, or pigment producing compound, such as a quinoid compound. The dye precursor is preferably an aromatic compound belonging to one of three major chemical families: the diamines, aminophenols(or aminonaphthols) and the phenols. The dye precursors can be used alone or in combination. At least one of the intermediates in the copolymerization must be an ortho- or para-diamine or aminophenol(primary intermediate). Examples of such are found in Section IV, below, and include p-phenylene-diamine(pPD), p-toluylene-diamine, chloro-p-phenylenediamine, p-aminophenol, o-aminophenol. 3,4-diaminotoluene; additional compounds are also described in US Patent No. 3,251,742, the contents of which are incorporated herein by reference. In one embodiment, the starting materials include not only the enzyme and a primary intermediate, but also a modifier(coupler) (or combination of modifiers), which modifier is typically a meta-diamine, meta-aminophenol, or a polyphenol. Examples of modifier

compounds include m-phenylene-diamine, 2,4-diaminoanisole, α-naphthol, hydroquinone, pyrocatechol, resorcinol. and 4-chlororesorcinol. The modifier then reacts with the primary intermediate in the presence of the laccase, converting it to a colored compound. In another embodiment, the laccase can be used with the primary intermediate directly, to oxidize it into a colored compound. In all cases, the dyeing process can be conducted with one or more primary intermediates, either alone or in combination with one or more modifiers. Amounts of components are in accordance with usual commercial amounts for similar components, and proportions of components may be varied accordingly.

The use of this laccase is an improvement over the more traditional use of H_2O_2, in that the latter can damage the hair, and its use usually requires a high pH, which is also damaging to the hair. In contrast, the reaction with laccase can be conducted at alkaline, neutral or even acidic pH, and the oxygen needed for oxidation comes from the air, rather than via harsh chemical oxidation. The result provided by the use of the *Myceliophthora* laccase is comparable to that achieved with use of H_2O_2, not only in color development, but also in wash stability and light fastness. An additional commercial advantage is that a single container package can be made containing both the laccase and the precursor, in an oxygen free atmosphere, which arrangement is not possible with the use of H_2O_2.

The present laccase can also be used for the polymerization of phenolic compounds present in liquids. An example of such utility is the treatment of juices, such as apple juice, so that the laccase will accelerate a precipitation of the phenolic compounds present in the juice, thereby producing a more stable juice. Such

applications have been described in Stutz, Fruit processing 7/93, 248-252, 1993; Maier et al., Dt. Lebensmittel-rindschau 86(5): 137-142, 1990; Dietrich et al., Fluss. Obst 57(2): 67-73, 1990,.

Laccases such as the *Myceliophthora* laccase are also useful in soil detoxification (Nannipieri et al., J. Environ. Qual. 20: 510-517,1991; Dec and Bollag, Arch. Environ. Contam. Toxicol. 19: 543-550, 1990).

The invention is further illustrated by the following non-limiting examples.

EXAMPLES

I. ISOLATION OF *MYCELIOPHTHORA THERMOPHILA* LACCASE GENE

A. MATERIALS AND METHODS

1. DNA Extraction and Hybridization analysis

Total cellular DNA is extracted from fungal cells of *Myceliophthora thermophila* strain E421 grown 24 hours in 25 ml of YEG medium (0.5% yeast extract, 2% glucose) using the following protocol: mycelia are collected by filtration through Miracloth (Calbiochem) and washed once with 25 ml of TE buffer. Excess buffer is drained from the mycelia which are subsequently frozen in liquid nitrogen. Frozen mycelia are ground to a fine powder in an electric coffee grinder,and the powder added to 20 ml of TE buffer and 5 ml of 20% SDS (w/v) in a disposable plastic centrifuge tube. The mixture is gently inverted several times to ensure mixing, and extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Sodium acetate (3M solution) is added to give a final concentration of 0.3 M and the nucleic acids are precipitated with 2.5 volumes of ice cold ethanol. The tubes are centrifuged at 15,000 x g for 30 minutes and the pellet is allowed to air-dry for 30 minutes before resuspending in 0.5 ml of TE buffer. DNase-

free ribonuclease A is added to a concentration of 100µg/ml and the mixture is incubated at 37°C for 30 minutes. Proteinase K (200µg/ml) is added and each tube is incubated an additional one hour at 37°C. Finally, each sample is extracted twice with phenol:chloroform:isoamyl alcohol before precipitating the DNA with sodium acetate and ethanol. DNA pellets are dried under vacuum, resuspended in TE buffer, and stored at 4°C.

Total cellular DNA samples from transformants and an untransformed control strain are analyzed by Southern hybridization. Approximately 5µg of DNA is digested with *Eco*RI and fractionated by size on a 1% agarose gel. The gel is photographed under short wavelength UV and soaked for 15 minutes in 0.5 M NaOH, 1.5 M NaCl followed by 15 minutes in 1 M Tris-HCl, pH 8, 1.5 M NaCl. DNA in the gel is transferred onto Zeta-Probe™ hybridization membrane (BioRad Laboratories) by capillary blotting in 20 X SSPE (R. W. Davis et al., Advanced Bacterial Genetics, A Manual for Genetic Engineering. Cold Spring Harbor Press. 1980) Membranes are baked for 2 hours at 80°C under vacuum and soaked for 2 hours in the following hybridization buffer at 45°C with gentle agitation: 5X SSPE, 35% formamide (v/v), 0.3% SCS, 200µg/ml denatured and sheared salmon testes DNA. The laccase-specific probe fragment (approx. 1.5 kb) encoding the 5'-portion of the *N. crassa lcc1* gene is amplified from *N. crassa* genomic DNA using standard PCR conditions (Perkin-Elmer Cetus, Emeryville, CA) with the following pair of primers: forward primer, 5' CGAGACTGATAACTGGCTTGG 3'; reverse primer, 5' ACGGCGCATTGTCAGGGAAGT 3'. The amplified DNA segment is first cloned into a TA-cloning vector (Invitrogen, Inc., San Diego, CA), then purified by agarose gel electrophoresis following digestion with *Eco*RI. The purified probe fragment is radiolabeled by nick translation with $\alpha[^{32}P]$dCTP(Amersham)

and added to the hybridization buffer at an activity of approximately 1 X 10^6 cpm per ml of buffer. the mixture is incubated overnight at 45°C in a shaking water bath. Following incubation, the membranes are washed once in 0.2 X SSPE with 0.1% SDS at 45°C followed by two washes in 0.2 X SSPE(no SDS) at the same temperature. The membranes are allowed to dry on paper towels for 15 minutes, then wrapped in Saran Wrap™ and exposed to x-ray film overnight at -70°C with intensifying screens(Kodak).

2. DNA Libraries and Identification of Laccase Clones

Genomic DNA libraries are constructed in the bacteriophage cloning vector λ-EMBL4(J.A.Sorge, in Vectors, A Survey of Molecular Cloning Vectors and Their Uses, Rodriguez et al., eds, pp.43-60, Butterworths, Boston, 1988). Briefly, total cellular DNA is partially digested with *Sau*3A and size-fractionated on low-melting point agarose gels. DNA fragments migrating between 9kb and 23 kb are excised and eluted from the gel using ß-agarase (New England Biolabs, Beverly MA). The eluted DNA fragments are ligated with *Bam*HI-cleaved and dephosphorylated λ-EMBL4 vector arms, and the ligation mixtures are packaged using commercial packaging extracts (Stratagene, LaJolla, CA). The packaged DNA libraries are plated and amplified on *Escherichia coli* K802 cells. Approximately 10,000-20,000 plaques from each library are screened by plaque-hybridization with the radiolabeled *lcc1* DNA fragment using the conditions described above. Plaques which give hybridization signals with the probe are purified twice on *E. coli* K802 cells, and DNA from the corresponding phage is purified from high titer lysates using a Qiagen Lambda kit(Qiagen, Inc., Chatsworth, CA).

3. Analysis of Laccase Genes

Restriction mapping of laccase clones is done using standard methods (Lewin, Genes. 2d ed., Wiley & Sons, 1985, New York). DNA sequencing is done with an Applied Biosystems Model 373A automated DNA Sequencer (Applied Biosystems, Inc., Foster City, CA) using the primer walking technique with dye-terminator chemistry (H. Giesecke et al., J. Virol. Methods 38: 47-60, 1992). Oligonucleotide sequencing primers are synthesized on an Applied Biosystems model 394 DNA/RNA Synthesizer.

B. RESULTS AND DISCUSSION

1. Identification of Laccase Gene Sequence

Total cellular DNA samples are prepared from the species *Neurospora crassa*, *Botrytis cinerea*, and *Myceliophthora*. Aliquots of these DNA preparations are digested with *Bam*HI and fractionated by agarose gel electrophoresis. DNA in the gel is blotted to a Zeta-Probe™ membrane filter (BioRad Laboratories, Hercules,CA) and probed under conditions of mild stringency with a radiolabeled fragment encoding a portion of the *N. crassa lcc1* gene, as described above. Laccase-specific sequences are detected in the genomes of *M. thermophila* and the *N. crassa* control, but not in the *B. cinerea* genomic DNA with this probe.

2. Cloning and Characterization of *Myceliophthora thermophila* Laccase (MtL) Gene

Approximately 20,000 plaques from a *M. thermophila* genomic DNA library constructed in a λ-EMBL4 cloning vector are screened. The library is composed of approximately 10,000 independent clones with inserts ranging in size from 9kb to 23kb. Assuming an average insert size of 10 kb and a total genome size of 4×10^7 bp for *M. thermophila*, this figure is about 2.5 times the number of clones required to represent the entire genome. Eight plaques are identified

that hybridized strongly to the *N. crassa* laccase gene probe. DNA is isolated from three of these, cleaved with *Eco*RI and analyzed by agarose gel electrophoresis and Southern hybridization. All three of these clones contain a 7.5 kb *Eco*RI fragment which hybridized to the laccase-specific probe. One of these *Eco*RI fragments is subcloned into pBR322 (Bolivar et al., Gene 2: 95-113, 1977) to generate plasmid pRaMB1. A restriction map of this DNA segment is shown in Fig. 1. The position of the laccase coding region on this clone is determined by hybridization with the *lcc1* gene fragment described above. Based on mapping data obtained, and an estimated size of the laccase protein of approximately 80 kdal, it is reasoned that the entire *M. thermophila* laccase coding region is contained with a 3.2 kb *Nhe*I-*Bgl*II segment which is then subcloned into pUC119(Viera and Messing, Methods Enzymol. 153: 3-11, 1987). The nucleotide sequence of this segment is determined using the primer walking method(Giesecke et al., *supra*). The nucleic acid sequence is shown in Figure 2 and SEQ ID NO: 1.

The deduced amino acid sequence of MtL is obtained on the basis of amino acid sequence homology with the *N. crassa* laccase. At the amino acid level, these two laccases share approximately 60% sequence identity. Similarity is highest in regions that correspond to the four histidines and one cysteine which are involved in the formation of the trinuclear copper cluster(Perry et al., J. Gen. Microbiol. 139: 1209-1218, 1993; Coll et al. Appl. Environ. Microbiol. 59: 4129-4135, 1993; Messerschmidt et al. J. Mol. Biol. 206: 513-530, 1989). There are 11 potential sites for N-linked glycosylation in the deduced amino acid sequence of MtL. the first 22 amino acids of MtL appear to comprise a canonical signal peptide with a predicted cleavage following an Ala residue (vonHeijne,J.Mol. Biol. 173:243-251, 1984).

Although the amino terminal sequence of the native MtL is unknown, the amino terminus of recombinant MtL produced in *A. oryzae* is blocked with a pyro-glutamate residue. Enzymatic removal of this residue followed by amino acid sequencing suggests that mature MtL begins with a Gln residue (position 1 in Figure 2; SEQ ID NO: 2). Thus, MtL is apparently synthesized as a 620 amino acid preproenzyme having a 22 amino acid signal peptide and propeptide of 25 residues. *Neurospora crassa* laccase(NcL) is processed similarly at its amino terminal end. In addition, NcL is also proteolytically processed at its C-terminus, resulting in the removal of 13 amino acids (Germann et al. J. Biol. Chem. 263: 885-896, 1988). The processing site is contained within the sequence Asp-Ser-Gly-Leu*Arg_{558} (where * designates the cleavage site). A similar sequence exists near the C-terminal end of MtL(Asp-Ser-Gly-Leu-Lys_{560}), suggesting the *Myceliophthora* enzyme may also be subject to C-terminal processing (Asp-Ser-Gly-Leu*Lys_{560}) which would remove 12 amino acids.

The positions of six introns (85, 84, 102, 72, 147, and 93 nucleotides) within the *lcc1* coding region are determined by comparing the deduced amino acid sequence of MtL to that of NcL and by applying the consensus rules for intron features in filamentous fungi (Gurr et al., *in* Gene Structure in Eukaryotic Microbes, J.R. Kinghorn, ed.) pp 93-139, IRL Press, Oxford, 1987). The 1860 nucleotides of coding sequence, excluding introns, are rich in guanosine and cytosine (65.5% G+C). The codon usage pattern for this gene reflects the DNA base composition in a strong bias(89.7%) for codons ending in G or C.

II. EXPRESSION OF *MYCELIOPHTHORA* LACCASE IN *ASPERGILLUS*

A. MATERIALS AND METHODS

1. Bacterial and Fungal Host Strains

Escherichia coli JM101(Messing et al., Nucl. Acids Res. 9:309-321, 1981) is used as a host for construction and routine propagation of laccase expression vectors in this study. Fungal hosts for laccase expression included the *Aspergillus niger* strains Bo-1, AB4.1 and AB1.13(Mattern et al., Mol. Gen. Genet. 234: 332-336), as well as a uridine-requiring(*pyrG*) mutant of the α-amylase-deficient *Aspergillus oryzae* strain HowB104.

2. Plasmids

Plasmid pRaMB2 is a pUC119 derivative which contains a 3.2 kb *Bgl*II-*Nhe*I fragment of *M. thermophila* genomic DNA encoding MtL. The vector pMWR is constructed by inserting the *A. oryzae* TAKA-amylase promoter and terminator elements from pTAKA17(Christensen et al., Bio/Technol. 6: 1419-1422, 1988; EP 238 023) into pUC18(Yanisch-Perron et al., Gene 33: 103-119, 1985). In this vector, there is a unique *Swa*I site at the end of the promoter element and a single *Nsi*I site at the beginning of the terminator for directional cloning of coding sequences. The cloning vehicle pUC518 is derived by inserting a small linker containing *Nsi*I, *Cla*I, *Xho*I, and *Bgl*II restriction sites between the adjacent *Bam*HI and *Xba*I sites of pUC118(Vieira and Messing, *supra*). Plasmid pToC68(WO 91/17243) contains the *A. oryzae* TAKA-amylase promoter and *A. niger glaA* terminator, and pToC90(WO 91/17243) carries the *A. nidulans amdS* gene.

3. Construction of Laccase Expression Vectors

The construction strategy for the laccase expression vector pRaMB5 is outlined in Figure 3. The promoter directing transcription of the laccase gene is obtained from the *A. oryzae* α-amylase (TAKA-amylase) gene (Christensen et al., *supra*), as well as the TAKA-amylase terminator region. The plasmid is constructed first by modifying pMWR3 by inserting a small linker which contains an *Apa*I site between

the *Swa*I and *Nsi*I sites, creating a plasmid called pMWR3-SAN. *Pfu*I polymerase-directed PCR (Stratagene, La Jolla, CA) is used to amplify a short DNA segment encoding the 5'-portion of MtL, from the start codon to an internal *Pst*I site (approximately 0.5 kb). The forward primer for this PCR reaction is designed to create an *Eco*RI site just upstream of the start codon. Next, the amplified fragment is digested with *Eco*RI and *Pst*I[during this step, the *Eco*RI site is made blunt by treatment with dNTPs and DNA polymerase I(Klenow fragment)] and purified by agarose gel electrophoresis. The 3' portion of the *M. thermophila* coding region is excised from pRaMB2 as a 2kb *Pst*I-*Apa*I fragment(this segment also contains approximately 110 bp from the 3'-untranslated region). These two fragments are combined with *Swa*I- and *Apa*I-cleaved pMWR3-SAN in a three-part ligation reaction to generate the laccase expression vector pRaMB5.

4. Transformation of *Aspergillus* host cells

Methods for co-transformation of *Aspergillus* strains are as described in Christensen et al., *supra*. For introduction of the laccase expression vectors into *A. oryzae*

HowB 104 *pyrG*, equal amounts (approximately 5 µg each) of laccase expression vector and one of the following plasmids are used: pPYRG (Fungal Genetics Stock Center, Kansas City, KS) which contains the *A. nidulans pyrG* gene(Oakley et al, Gene 61385-399, 1987); pSO2 which harbors the clones *A. oryzae pyrG* gene; pPRYG24 which contains the *A. ficuum*(=*A. niger*)*pyrG* gene. Protrophic(Pyr+) transformants are selected on *Aspergillus* minimal medium (Rowlands and Turner, Mol. Gen. Genet. 126: 201-216, 1973), and the transformants are transformants are screened for the ability to produce laccase on minimal medium containing 1 mM 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)[ABTS]. Cells which

secrete active laccase oxidize the ABTS, producing a green halo surrounding the colony. Lastly, *A. niger* Bo-1 protoplasts are co-transformed using equal amounts (approximately 5µg each) of laccase expression vector and pToC90 which contains the *A. nidulans amdS* (acetamidase) gene (Hynes et al., Mol. Cell Biol. 3: 1430-1439, 1983. AmdS+ transformants are selected on Cove minimal medium (Cove, Biochim. Biophys. Acta 113: 51-56, 1966) with 1% glucose as the carbon source and acetamide as the sole nitrogen source and screened for laccase expression on cove medium with 1 mM ABTS.

5. Analysis of Laccase-Producing Transformants

Transformants which produce laccase activity on agar plates are purified twice through conidiospores and spore suspensions in sterile 0.01% Tween-80 are made from each. The density of spores in each suspension is estimated spectrophotometrically (A_{595} nm). Approximately 0.5 absorbance units of spores are used to inoculate 25 ml of ASPO4 or MY50 medium in 125 ml plastic flasks. The cultures are incubated at 37°C with vigorous aeration (approximately 200 rpm) for four to five days. Culture broths are harvested by centrifugation and the amount of laccase activity in the supernatant is determined using syringaldazine as a substrate. Briefly, 800 µl of assay buffer (25 mM sodium acetate, pH 5.5, 40 µM $CuSo_4$) is mixed with 20 µl of culture supernatant and 60 µl of 0.28 mM syringaldazine (Sigma Chemical Co., St. Louis, MO) in 50% ETOH. The absorbance at 530 nm is measured over time in a Genesys 5 UV-vis spectrophotometer (Milton-Roy). One laccase unit(LACU) is defined as the amount of enzyme which oxidizes one µmole of substrate per minute at room temperature. SDS-polyacrylamide gel electrophoresis(PAGE) is done using precast 10-27% gradient gels from Novex(San

Diego, CA). Protein bands are developed using Coomassie Brilliant Blue(Sigma).

B.RESULTS AND DISCUSSION

1. Expression of *Myceliophthora* laccase

Laccase-producing transformants are detected by incorporation of ABTS into selective media. Using *pyrG* or *amdS* as the selectable marker, co-transformation frequencies vary from about 30% to 70%. Heterologous expression of MtL appears to be highest in *A. oryzae* transformants. Furthermore, production appears to be better in ASPO4 medium compared to MY50, although the reasons for this are unknown. SDS-PAGE analysis of culture broth samples shows a prominent laccase band at approximately 80 kdal, which is similar to the size of the native enzyme purified from *M. thermophila*. Similar analysis of the culture filtrates from *A. niger* Bo-transformants indicate that the laccase band is obscured by very intense glucoamylase and acid-stable amylase protein bands. Results are shown in Table 1.

Table 1. MtL expression among selected *A. oryzae* and *A. niger* transformants

HOST STRAIN	TRANSFORMANT	TRANSFORMING DNAS	MTLACU/ML	
			ASPO4	MY50
A. oryzae HowB104	untransformed	none	0.00	0.00
pyrG	RaMB5.15	pRaMB5+pPYRG	0.85	0.29
	RaMB5.30	pRaMB5+pPYRG	0.71	0.87
	RaMB5.33	pRaMB5+pPYRG	0.60	0.26
	RaMB5.108	pRaMB5+PSO2	0.68	0.19
	RaMB5.111	pRaMB5+PSO2	0.70	0.17
	RaMB5.121	pRaMB5+PSO2	0.49	0.20
	RaMB5.142	pRaMB5+PSO2	0.54	0.04
A. Niger Bo-1	untransformed	none	0.00	0.00
	RaMB5.1	pRaMB5+pToC90	n.d.	0.20
	RaMB5.25	pRaMB5+pToC90	n.d.	0.09
	RaMB5.49	pRaMB5+pToC90	n.d.	0.06
	RaMB5.51	pRaMB5+pToC90	n.d.	0.12
	RaMB5.53	pRaMB5+pToC90	n.d.	0.21
	RaMB5.62	pRaMB5+pToC90	n.d.	0.16

n.d.= not determined

2. Expression in the presence or absence of excess copper

A 1 ml aliquot of a spore suspension of *Aspergillus oryzae* transformant HowB104-pRaMB5.30(approximately 10^9 spores/ml) is added aseptically to a 500 ml shake flask containing 100 ml of sterile shake flask medium (maltose, 50g/l; $MgSO_4 \cdot 7H_2O$, 2g/l; KH_2PO_4, 10g/l; K_2SO_4, 2g/l; $CaCl_2 \cdot 2H_2O$ 0.5 g/l; Citric acid, 2g/l; yeast extract, 10g/l; trace metals[$ZnSO_4 \cdot 7H_2O$, 14.3 g/l; $CuSO_4 \cdot 5H_2O$, 2.5 g/l; $NiCl_2 \cdot 6H_2O$, 0.5 g/l; $FeSO_4 \cdot 7H_2O$, 13.8 g/l, $MnSO_4 \cdot H_2O$, 8.5 g/l; citric acid, 3.0 g/l], 0.5 ml/l; urea, 2g/l, made with tap water and adjusted to pH 6.0 before autoclaving), and incubated at 37°C on a rotary shaker at 200 rpm for 18 hours. 50 ml of this culture is aseptically transferred to a 3 liter fermentor containing 1.8 liters of the fermentor media ($MgSO_4 \cdot 7H_2O$, 2g/l; KH_2PO_4, 2g/l; citric acid 4g/l; K_2SO_4, 3g/l;$CaCl_2 \cdot 2H_2O$, 2g/l; trace metals, 0.5 ml/l; pluronic antifoam, 1ml/l). The fermentor temperature is maintained at 34°C by the circulation of cooling water through the fermentor jacket. Sterile air is sparged through the fermentor at a rate of 1.8 liter/min (1v/v/m). The agitiation rate is maintained between 600 and 1300 rpm at approximately the minimum level required to maintain the dissolved oxygen level in the culture above 20%. Sterile feed (Nutriose 725[maltose syrup], 225 g/l; urea, 30 g/l; yeast extract, 15 g/l; pluronic antifoam, 1.5 ml/l, made up with distilled water and autoclaved) is added to the fermentor by use of a peristaltic pump. The feed rate profile during the fermentation is as follows: 30 g of feed is added initially before inoculation; 0-24 h, 2 g/l h; 24-48 h, 4 g/l h, 48h-end, 6 g/l.

Copper is made as a 400X stock in water or a suitable buffer, filter sterilized and added aseptically to the tank

to a final level of 0.5 mM. The fermentation described above is also conducted without the addition of copper supplement to tha tank medium. Samples for enzyme activity determination are withdrawn and filtered through Miracloth to remove mycelia. These samples are assayed for laccase activity by the LACU assay described above. Laccase activity is found to increase continuously during the course of the fermentation, with a value of approximately 45 LACU/ml achieved after 180 hours in the fermentation containing excess copper. At a specific activity of 22 LACU/mg, this corresponds to 2g/l of recombinant laccase expressed. On the other hand, the maximum laccase activity achieved in the fermentation without copper supplement is approximately 10 LACU/ml after 170 hours, or about 25% of that found in the presence of additional copper.

III. PURIFICATION AND CHARACTERIZATION OF *MYCELIOPTHORA* LACCASE

A. MATERIALS AND METHODS

1. Materials

Chemicals used as buffers and substrates are commercial products of at least reagent grade. Endo/N-glycosidase F and pyroglutamate amino peptidase are purchased from Boehringer Mannheim. Chromatography is performed on either a Pharmacia FPLC or a conventional low pressure system. Spectroscopic assays are conducted on either a spectrophotometer(Shimadzu PC160) or a microplate reader(Molecular Devices). Britton & Robinson(B&R) buffers are prepared according to the protocol described in Quelle, Biochemisches Taschenbuch, H.M. Raven, II. Teil, S.93 u. 102, 1964.

2. Enzymatic Assay

Laccase activity is determined by syringaldazine oxidation at 30°C in a 1-cm quartz cuvette. 60µl

syringaldazine stock solution (0.28 mM in 50% ethanol) and 20 µl sample are mixed with 0.8 ml preheated buffer solution. The oxidation is monitored at 530nm over 5 minutes. The activity is expressed as µmole substrate oxidized per minute. B&R buffers with various pHs are used. The activity unit is referred to here as "SOU". A buffer of 25 mM sodium acetate, 40 µM $CuSO_4$, pH 5.5, is also used to determine the activity, which is referred to as LACU, as defined above. 2,2'-azinobis(3-ethylbenzo thiazoline-6-sulfonic acid) (ABTS) oxidation assays are done using 0.4 mM ABTS, B&R buffer, pH 4.1, at room temperature by monitoring ΔA_{405}. An ABTS oxidase activity overlay assay is performed by pouring cooled ABTS-agarose(0.05 g ABTS, 1 g agarose, 50 ml H_2O, heated to dissolve agarose) over a native IEF gel and incubating at room temperature. Thermostability analysis of the laccase(r-MtL) is performed using samples that have 3 SOU activity pre-incubated in B&R buffer, pH 6, at various temperatures. Samples are assayed after a 400-fold dilution into the same buffer at room temperature.

3. Purification from a fermentor broth

3.7 liters of cheese-cloth filtered broth (pH 7.6, 16 mS) is filtered through Whatman #2 filter paper. The broth is concentrated on a Spiral Concentrator (Amicon) with a S1Y100 membrane (MWCO:100) from 3700 ml to 200 ml. The concentrate is adjusted to 0.75 mS by diluting it in water and reconcentrated on S1Y100 to 170 ml. The washed and concentrated broth has a dense greenish color.

The broth is frozen overnight at -20°C, thawed the next day and loaded onto a Q-sepharose XK26 column (120 ml), pre-equilibrated with 10 mM Tris, pH 7.5, 0.7 mS(Buffer A). The blue laccase band migrates slowing down the column during loading. One group of blue fractions runs through the column after loading and washing by Buffer A. A second group eluted during the linear gradient with Buffer B

(Buffer A plus 2 M NaCl). Some brown material with no laccase activity is eluted out later with 1 M NaOH. SDS-PAGE analysis shows that this preparation results in pure laccase.

4. Analyses of amino acid content, extent of glycosylation, and N-terminal sequence

N-terminal sequencing is performed on an ABI 476A sequencer. Total amino acid analysis, from which the extinction coefficient of r-MtL is determined, is performed on a HP AminoQuant instrument. Deglycosylation is done using endo/N-glucosidase F according to the manufacturer's instructions and carbohydrate content is estimated by mobility difference as determined on SDS-PAGE. N-terminus de-blocking with pyroglutamate amino peptidase is carried out according to manufacturer's instructions. About 80µg r-MtL is treated with 4 µg peptidase with or without the presence of 1 M urea or 0.1 M guanidine HCl before being blotted on a PVDF membrane for sequencing. About 20 pmol de-blocked protein is obtained and sequenced.

SDS-PAGE and native IEF analysis are performed on either a Novex cell or a Mini Protean II and a Model 111 Mini IEF cells (Bio-Rad). Gel filtration analyses are done on a Sephacryl S-300(Pharmacia), from which the native MW is estimated by using Blue Dextran (2000 kdal), bovine IgG (158 kdal), bovine serum albumin (66 kdal), ovalbumin (45 kdal) and horse heart myoglobin(17 kdal) to calibrate the column.

B. RESULTS AND DISCUSSION

1. Purification and characterization of r-MtL from a fermentor broth

From 3.7 l of fermentor broth, about 2-3 g of r-MtL are isolated. Initial concentration using a membrane with MWCO of 100 kdal removed significant amounts of brown material and small contaminant proteins. The low affinity of r-MtL toward Q-Sepharose matrix equilibrated with 10 mM Tris, pH

7.5, facilitates its separation from other more acidic and more tightly bound impurities. As shown by SDS-PAGE, this preparation resulted in essentially pure laccase for the most active fractions located around the peak. Other less active fractions can be further purified on either Mono-Q with a shallower gradient or a gel filtration column, such as S-300, from which the contaminants are separated due to their smaller MW. An overall 18-fold purification and a recovery of 67% are achieved. As discussed below, the existence of two elution bands of r-MtL on Q-Sepharose chromatogram is probably due to a differential glycosylation.

The purified r-MtL shows a MW of 100-140 kdal on S-300 gel filtration and a MW of 85 kdal on SDS-PAGE. The increase of r-MtL mobility on SDS-PAGE after deglycosylation suggests that carbohydrates account for 14% of its total mass. Native IEF shows a major band at pI ~4.2 that is active in ABTS overlay assay.

Directly sequencing the N-terminus of the purified r-MtL from samples either in desalted solution or on PVDF membrane are unsuccessful. However, treatment of r-MtL with pyroglutamate amino peptidase yielded a protein with deblocked N-terminus. This suggests the processing of a propeptide during the maturation of r-MtL, a posttranslational event similar to that of *N. crassa* laccase but not found in other laccases such as *Rhizoctonia solani*. The proposed scheme is outlined below.

```
MKSFISAATLWIVGILTPSVAAAPPSTEPQRDLLVPITEREEAAVKARQQSCNTPS
|<-putative signal peptide-> |<-    putative propeptide    ->|<-N-terminus
```

The spectrum of the blue r-MtL has absorption maxima at 276 and 589 nm.

The activity of the laccase is tested by using either syringaldazine and ABTS as substrates. Expressed as per Abs_{276} or per mg, the laccase has a value of 20 or 45 units for SOU at pH 6.5, respectively. The LACU assay yields a value of 10 or 22 units per Abs_{276} or per mg.

The pH profile of r-MtL activity is quite close to that of the wild type, with an optimal pH of 6.5. The upper temperature limit for retaining full activity after a 20 minute preincubation observed for r-MtL is approximately 60°C. The purified r-MtL shows no activity loss over a 5 week storage frozen in Q-sepharose elution buffer at -20°C.

When comparing the two forms of r-MtL obtained from the fermentor broth isolated on Q-Sepharose, there are no significant differences seen in terms of SDS-PAGE, native PAGE, native IEF, S-300 gel filtration, UV-visible spectrum, specific activity towards syringaldazine and ABTS, and deblocked N-terminus sequencing measurements. Likely, the different elution pattern on Q-Sepharose arises from some sort of differential glycosylation.

IV. USE OF *MYCELIOPHTHORA* LACCASE IN DYEING HAIR

The dyeing effect of *Myceliophthora* laccase is tested on various dye precursors and further on 0.1% p-phenylenediamine compared with a number of modifiers.

Materials:

Dye precursors:

0.1 % p-phenylene-diamine in 0.1 M K-phosphate buffer, pH=7.0)

0.1 % o-aminophenol in 0.1 M K-phosphate buffer, pH=7.0)

Enzymes:

Recombinant *Myceliophthora thermophila* laccase, 16 LACU/ml (in final dye solution).

Equipment:

Datacolor Textflash 2000 (CIE-Lab)

Assessment of the hair color

The quantitative color of the hair tresses is determined on a Datacolor Textflash 2000 by the use of CIE-Lab parameters L* ("0"=black and "100"=white) combined with a* ("-"=green and "+"=red).

Results:

Dyeing effect

Tresses of blond European hair (1 gram) are used for testing *Myceliophthora thermophila* laccase in the context of oxidative hair dyeing. p-phenylene diamine and o-aminophenol are used as the dye precursors.

Hair dyeing

4 ml dye precursor solution is mixed with 1 ml laccase on a Whirley mixer, applied to the hair tresses and kept at 30°C for 60 minutes. The hair tresses are then rinsed with running water for about 3 minutes, pressed between two fingers, combed, and air dried.

The results of the dyeing effect test are displayed below in Table 1 and 2.

Table 1

o-aminophenol	enzyme	L*	a*
Untreated blond hair	-	70.3	2.3
Laccase	+	57.7	15.3

: 0=black, 100=white a: -=green, +=red

Table 2

p-phenylenediamine	enzyme	L*	a*
Untreated blond hair	-	70.3	2.3
1.0 ml laccase	+	29.1	4.1

L*: 0=black, 100=white a*: -=green, +=red

Result of test:

From Table 1 and 2 it can be seen that the *Myceliophthora thermophila* laccase can be used for oxidative dyeing of hair.

Deposit of Biological Materials

The following biological materials have been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection, Northern Regional Research Center, 1815 University Street, Peoria, Illinois, 61604 on May 25, 1994, and given the following accession number.

Deposit	Accession Number
E. coli JM101 containing pRaMB5	NRRL B-21261

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Novo Nordisk Biotech, Inc.
(B) STREET: 1445 Drew Avenue
(C) CITY: Davis, California
(D) COUNTRY: United States of America
(E) POSTAL CODE (ZIP): 95616-4880
(F) TELEPHONE: (916) 757-8100
(G) TELEFAX: (916) 758-0317

(i) APPLICANT:

(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsværd
(D) COUNTRY: Denmark
(E) POSTAL CODE (ZIP): DK-2880
(F) TELEPHONE: +45 4444 8888
(G) TELEFAX: +45 4449 3256

(ii) TITLE OF INVENTION: PURIFIED MYCELIOPHTHORA LACCASES AND NUCLEIC ACIDS ENCFODING SAME

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Novo Nordisk of North America, Inc.
(B) STREET: 405 Lexington Avenue, Suite 6400
(C) CITY and STATE: New York, New York
(D) COUNTRY: U.S.A.
(E) ZIP: 10174-6401

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: to be assigned
(B) FILING DATE: 31-May-1995
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/253,781
(B) FILING DATE: 03-June-1994

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Lowney, Karen A.
(B) REGISTRATION NUMBER: 31,274
(C) REFERENCE/DOCKET NUMBER: 4184.204-WO

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 212 867 0123
(B) TELEFAX: 212 867 0298

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3187 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Myceliophthora thermophila

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 833...917

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 996...1077

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1090...1188

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1261...1332

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2305...2451

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2521...2613

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join (587..832, 918..995, 1078..1089, 1189..1260, 1333..2304, 2452..2520, 2614..3024)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
GCTAGCTTCT TTGGTCACCG TCGTTTTCGC CCGCCCCCTC CCTCCTTCAA CCCCCTGAGT       60

AGTCGGCTAA GCGATCCTCA ATCTGGTCTT GTGAGGTCAC GTCCTCCAGC AGATGACAGT      120

TCATCGAGCG AGTGATCTCC ACCACCCAGA AGGGAGGGGG GATGCGCGCA TGCTCCAACA      180

TACCCTGGTG TCGCTAGAGA CGTCGCGGCA TCAGCCTTTT CATCACACCG AGCACGTCCA      240

CGGACCGGCT CCTTTCACCC CCGCGTCCTC CGGAGGATTG AGTCACGATA TTTCGGGATG      300

TGGGAAGGGG GAGAGAAAGG AGGGGGGAGG GGCGGAAACA TGTTGGATAC GAGCTGCGCC      360

CCTTTTCCAA CATCGAGAAC AGGAAGTCGT TGGTGTCGGC CGTAATGTCT ATAAAACGAG      420

GCTCCTTCTC GTCGTCGACT TGTCTCAGGT TCTCTCTCTC GTCCACACCA AGCCAGTCTT      480

GCCTGAGCCA CCTGAGCCAC CTTCAACTCA TCATCTTCAG TCAAGTCGTT CATTGACATT      540

GTGTCTCTC TTTCTATCGAG TCGGCTTCCC GGCCCTTCAC CACAAC ATG AAG TCC          595
                                                    Met Lys Ser
                                                    1

TTC ATC AGC GCC GCG ACG CTT TTG GTG GGC ATT CTC ACC CCT AGC GTT        643
Phe Ile Ser Ala Ala Thr Leu Leu Val Gly Ile Leu Thr Pro Ser Val
    5                   10                  15

GCT GCT GCC CCT CCA TCC ACC CCT GAG CAG CGC GAC CTG CTC GTC CCG        691
Ala Ala Ala Pro Pro Ser Thr Pro Glu Gln Arg Asp Leu Leu Val Pro
20                  25                  30                  35
```

```
ATC ACG GAG AGG GAG GAG GCA GCC GTG AAG GCT CGC CAG CAG AGC TGC          739
Ile Thr Glu Arg Glu Glu Ala Ala Val Lys Ala Arg Gln Gln Ser Cys
                40                  45                  50

AAC ACC CCC AGC AAC CGG GCG TGC TGG ACT GAC GGA TAC GAC ATC AAC          787
Asn Thr Pro Ser Asn Arg Ala Cys Trp Thr Asp Gly Tyr Asp Ile Asn
            55                  60                  65

ACC GAC TAC GAA GTG GAC AGC CCG GAC ACG GGT GTT GTT CGG CCG              832
Thr Asp Tyr Glu Val Asp Ser Pro Asp Thr Gly Val Val Arg Pro
        70                  75                  80

GTGAGTGCTC TCGTTAATTA CGCTTCGGCG AGTTGCGCAG ATATATTAAA TACTGCAAAC        892

CTAAGCAGGA GCTGACATGC GACAG TAC ACT CTG ACT CTC ACC GAA GTC GAC          944
                            Tyr Thr Leu Thr Leu Thr Glu Val Asp
                                85                  90

AAC TGG ACC GGA CCT GAT GGC GTC GTC AAG GAG AAG GTC ATG CTG GTT          992
Asn Trp Thr Gly Pro Asp Gly Val Val Lys Glu Lys Val Met Leu Val
            95                  100                 105

AAC GTACGGCACC CCTTTTCTTG TCCTAGGATC TGGGTGATGT GCGTCGTTGC              1045
Asn

CCCTGAGAGA CTGACCGAGC CTTTGGCTGC AG AAT AGT ATA ATC GTAATTAATT          1099
                                  Asn Ser Ile Ile
                                      110

ATACCGCCCT GCCTCCAGCA GCCCCAGCAG CTCGAGAAGG GTATCTGAAG TTAGTCAGGC      1159

CTGCTGACCT GACCGGGGCC AACCCATAG GGA CCA ACA ATC TTT GCG GAC TGG         1212
                               Gly Pro Thr Ile Phe Ala Asp Trp
                                   115                 120

GGC GAC ACG ATC CAG GTA ACG GTC ATC AAC AAC CTC GAG ACC AAC GGC         1260
Gly Asp Thr Ile Gln Val Thr Val Ile Asn Asn Leu Glu Thr Asn Gly
            125                 130                 135

GTATGTCTGC TGCTTGCTCT CTTGCTCTCC TCGTCCGCGA CTAATAATAA TATCAACTCG      1320

TGTGGAAAAC AG ACG TCG ATC CAC TGG CAC GGA CTG CAC CAG AAG GGC           1368
              Thr Ser Ile His Trp His Gly Leu His Gln Lys Gly
                  140                 145

ACC AAC CTG CAC GAC GGC GCC AAC GGT ATC ACC GAG TGC CCG ATC CCC         1416
Thr Asn Leu His Asp Gly Ala Asn Gly Ile Thr Glu Cys Pro Ile Pro
    150                 155                 160

CCC AAG GGA GGG AGG AAG GTG TAC CGG TTC AAG GCT CAG CAG TAC GGG         1464
Pro Lys Gly Gly Arg Lys Val Tyr Arg Phe Lys Ala Gln Gln Tyr Gly
165                 170                 175                 180

ACG AGC TGG TAC CAC TCG CAC TTC TCG GCC CAG TAC GGC AAC GGC GTG         1512
Thr Ser Trp Tyr His Ser His Phe Ser Ala Gln Tyr Gly Asn Gly Val
                185                 190                 195

GTC GGG GCC ATT CAG ATC AAC GGA CCG GCC TCG CTG CCG TAC GAC ACC         1560
Val Gly Ala Ile Gln Ile Asn Gly Pro Ala Ser Leu Pro Tyr Asp Thr
            200                 205                 210

GAC CTG GGT GTG TTC CCC ATC AGC GAC TAC TAC TAC AGC TCG GCC GAC         1608
Asp Leu Gly Val Phe Pro Ile Ser Asp Tyr Tyr Tyr Ser Ser Ala Asp
        215                 220                 225
```

```
GAG CTG GTG GAA CTC ACC AAG AAC TCG GGC GCG CCC TTC AGC GAC AAC        1656
Glu Leu Val Glu Leu Thr Lys Asn Ser Gly Ala Pro Phe Ser Asp Asn
230                 235                 240                 245

GTC CTG TTC AAC GGC ACG GCC AAG CAC CCG GAG ACG GGC GAG GGC GAG        1704
Val Leu Phe Asn Gly Thr Ala Lys His Pro Glu Thr Gly Glu Gly Glu
                250                 255                 260

TAC GCC AAC GTG ACG CTC ACC CCG GGC CGG CGG CAC CGC CTG CGC CTG        1752
Tyr Ala Asn Val Thr Leu Thr Pro Gly Arg Arg His Arg Leu Arg Leu
            265                 270                 275

ATC AAC ACG TCG GTC GAG AAC CAC TTC CAG GTC TCG CTC GTC AAC CAC        1800
Ile Asn Thr Ser Val Glu Asn His Phe Gln Val Ser Leu Val Asn His
        280                 285                 290

ACC ATG TGC ATC ATC GCC GCC GAC ATG GTG CCC GTC AAC GCC ATG ACG        1848
Thr Met Cys Ile Ile Ala Ala Asp Met Val Pro Val Asn Ala Met Thr
    295                 300                 305

GTC GAC AGC CTC TTC CTC GGC GTC GGC CAG CGT TAC GAT GTC GTC ATC        1896
Val Asp Ser Leu Phe Leu Gly Val Gly Gln Arg Tyr Asp Val Val Ile
310                 315                 320                 325

GAA GCC AAC CGA ACG CCC GGG AAC TAC TGG TTT AAC GTC ACA TTT GGC        1944
Glu Ala Asn Arg Thr Pro Gly Asn Tyr Trp Phe Asn Val Thr Phe Gly
                330                 335                 340

GGC GGC CTG CTC TGC GGC GGC TCC AGG AAT CCC TAC CCG GCC GCC ATC        1992
Gly Gly Leu Leu Cys Gly Gly Ser Arg Asn Pro Tyr Pro Ala Ala Ile
            345                 350                 355

TTC CAC TAC GCC GGC GCC CCC GGC GGC CCG CCC ACG GAC GAG GGC AAG        2040
Phe His Tyr Ala Gly Ala Pro Gly Gly Pro Pro Thr Asp Glu Gly Lys
        360                 365                 370

GCC CCG GTC GAC CAC AAC TGC CTG GAC CTC CCC AAC CTC AAG CCC GTC        2088
Ala Pro Val Asp His Asn Cys Leu Asp Leu Pro Asn Leu Lys Pro Val
    375                 380                 385

GTG GCC CGC GAC GTG CCC CTG AGC GGC TTC GCC AAG CGG GCC GAC AAC        2136
Val Ala Arg Asp Val Pro Leu Ser Gly Phe Ala Lys Arg Ala Asp Asn
390                 395                 400                 405

ACG CTC GAC GTC ACC CTC GAC ACC ACG GGC ACG CCC CTG TTC GTC TGG        2184
Thr Leu Asp Val Thr Leu Asp Thr Thr Gly Thr Pro Leu Phe Val Trp
                410                 415                 420

AAG GTC AAC GGC AGC GCC ATC AAC ATC GAC TGG GGG AGG GCC GTC GTC        2232
Lys Val Asn Gly Ser Ala Ile Asn Ile Asp Trp Gly Arg Ala Val Val
            425                 430                 435

GAC TAC GTC CTC ACG CAG AAC ACC AGC TTC CCA CCC GGG TAC AAC ATT        2280
Asp Tyr Val Leu Thr Gln Asn Thr Ser Phe Pro Pro Gly Tyr Asn Ile
        440                 445                 450

GTC GAG GTG AAC GGA GCT GAT CAG GTAAGAAAAA GGGGACCGCA GGGGTGCTGC       2334
Val Glu Val Asn Gly Ala Asp Gln
    455                 460

TGCAAGTACA CCTTGCTCGC CCTCCTGTTC TTCCTTAATA ACTACCTCCC AACCCTCCCC      2394

CCTAATTAAT TCACTTTAAA GGCCGATCAA GACTGACCGA GCCCCCTCTC TTTGCAG         2451

TGG TCG TAC TGG TTG ATC GAG AAC GAT CCC GGC GCA CCT TTC ACC CTA        2499
Trp Ser Tyr Trp Leu Ile Glu Asn Asp Pro Gly Ala Pro Phe Thr Leu
            465                 470                 475
```

```
CCG CAT CCG ATG CAC CTG CAC GTAAGTTGGA TACATATATA TATATATATA          2550
Pro His Pro Met His Leu His
        480

TACATTGCTT TCCTGGCTCG CTCCCTTAAA TAAAATTAAA TAACCAAAAA TAACAAAAAA     2610

AAG GGC CAC GAC TTT TAC GTG CTG GGC CGC TCG CCC GAC GAG TCG CCG       2658
    Gly His Asp Phe Tyr Val Leu Gly Arg Ser Pro Asp Glu Ser Pro
    485                 490                 495

GCA TCC AAC GAG CGG CAC GTG TTC GAT CCG GCG CGG GAC GCG GGC CTG       2706
Ala Ser Asn Glu Arg His Val Phe Asp Pro Ala Arg Asp Ala Gly Leu
500                 505                 510                 515

CTG AGC GGG GCC AAC CCT GTG CGG CGG GAC GTG TCG ATG CTG CCG GCG       2754
Leu Ser Gly Ala Asn Pro Val Arg Arg Asp Val Ser Met Leu Pro Ala
                520                 525                 530

TTC GGG TGG GTG GTG CTG TCC TTC CGG GCC GAC AAC CCG GGC GCC TGG       2802
Phe Gly Trp Val Val Leu Ser Phe Arg Ala Asp Asn Pro Gly Ala Trp
            535                 540                 545

CTG TTC CAC TGC CAC ATC GCC TGG CAC GTC TCG GGC GGC CTG GGC GTC       2850
Leu Phe His Cys His Ile Ala Trp His Val Ser Gly Gly Leu Gly Val
        550                 555                 560

GTC TAC CTC GAG CGC GCC GAC GAC CTG CGC GGG GCC GTC TCG GAC GCC       2898
Val Tyr Leu Glu Arg Ala Asp Asp Leu Arg Gly Ala Val Ser Asp Ala
    565                 570                 575

GAC GCC GAC GAC CTC GAC CGC CTC TGC GCC GAC TGG CGC CGC TAC TGG       2946
Asp Ala Asp Asp Leu Asp Arg Leu Cys Ala Asp Trp Arg Arg Tyr Trp
        580                 585                 590

CCT ACC AAC CCC TAC CCC AAG TCC GAC TCG GGC CTC AAA CAC CGC TGG       2994
Pro Thr Asn Pro Tyr Pro Lys Ser Asp Ser Gly Leu Lys His Arg Trp
    595                 600                 605

GTC GAG GAG GGC GAG TGG CTG GTC AAG GCG TGAGCGAAGG AGGAAAAAGG        3044
Val Glu Glu Gly Glu Trp Leu Val Lys Ala
610                 615

AAACAAAGAG GGGGGGGGGG GCTAGTTCCT ATTTTTGCTT TTTTTTTTTG TTCTTGTCCT    3104

TGTGCTGGCG GTTCCCTGGT AAAGGAGAAG GGGGCCCCAA GTTCGAGTGG GTGTGTGATC    3164

GGGTAAATAT TATCAAGAGA TCT                                            3187
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 620 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Myceliophthora thermophila

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Lys Ser Phe Ile Ser Ala Ala Thr Leu Leu Val Gly Ile Leu Thr
1               5                   10                  15
```

```
Pro Ser Val Ala Ala Ala Pro Pro Ser Thr Pro Glu Gln Arg Asp Leu
            20                  25                  30

Leu Val Pro Ile Thr Glu Arg Glu Glu Ala Ala Val Lys Ala Arg Gln
        35                  40                  45

Gln Ser Cys Asn Thr Pro Ser Asn Arg Ala Cys Trp Thr Asp Gly Tyr
    50                  55                  60

Asp Ile Asn Thr Asp Tyr Glu Val Asp Ser Pro Asp Thr Gly Val Val
65                  70                  75                  80

Arg Pro Tyr Thr Leu Thr Leu Thr Glu Val Asp Asn Trp Thr Gly Pro
                85                  90                  95

Asp Gly Val Val Lys Glu Lys Val Met Leu Val Asn Asn Ser Ile Ile
            100                 105                 110

Gly Pro Thr Ile Phe Ala Asp Trp Gly Asp Thr Ile Gln Val Thr Val
        115                 120                 125

Ile Asn Asn Leu Glu Thr Asn Gly Thr Ser Ile His Trp His Gly Leu
    130                 135                 140

His Gln Lys Gly Thr Asn Leu His Asp Gly Ala Asn Gly Ile Thr Glu
145                 150                 155                 160

Cys Pro Ile Pro Pro Lys Gly Gly Arg Lys Val Tyr Arg Phe Lys Ala
                165                 170                 175

Gln Gln Tyr Gly Thr Ser Trp Tyr His Ser His Phe Ser Ala Gln Tyr
            180                 185                 190

Gly Asn Gly Val Val Gly Ala Ile Gln Ile Asn Gly Pro Ala Ser Leu
        195                 200                 205

Pro Tyr Asp Thr Asp Leu Gly Val Phe Pro Ile Ser Asp Tyr Tyr Tyr
    210                 215                 220

Ser Ser Ala Asp Glu Leu Val Glu Leu Thr Lys Asn Ser Gly Ala Pro
225                 230                 235                 240

Phe Ser Asp Asn Val Leu Phe Asn Gly Thr Ala Lys His Pro Glu Thr
                245                 250                 255

Gly Glu Gly Glu Tyr Ala Asn Val Thr Leu Thr Pro Gly Arg Arg His
            260                 265                 270

Arg Leu Arg Leu Ile Asn Thr Ser Val Glu Asn His Phe Gln Val Ser
        275                 280                 285

Leu Val Asn His Thr Met Cys Ile Ile Ala Ala Asp Met Val Pro Val
    290                 295                 300

Asn Ala Met Thr Val Asp Ser Leu Phe Leu Gly Val Gly Gln Arg Tyr
305                 310                 315                 320

Asp Val Val Ile Glu Ala Asn Arg Thr Pro Gly Asn Tyr Trp Phe Asn
                325                 330                 335

Val Thr Phe Gly Gly Gly Leu Leu Cys Gly Gly Ser Arg Asn Pro Tyr
            340                 345                 350

Pro Ala Ala Ile Phe His Tyr Ala Gly Ala Pro Gly Gly Pro Pro Thr
        355                 360                 365
```

```
Asp Glu Gly Lys Ala Pro Val Asp His Asn Cys Leu Asp Leu Pro Asn
    370                 375                 380

Leu Lys Pro Val Val Ala Arg Asp Val Pro Leu Ser Gly Phe Ala Lys
385                 390                 395                 400

Arg Ala Asp Asn Thr Leu Asp Val Thr Leu Asp Thr Thr Gly Thr Pro
                405                 410                 415

Leu Phe Val Trp Lys Val Asn Gly Ser Ala Ile Asn Ile Asp Trp Gly
            420                 425                 430

Arg Ala Val Val Asp Tyr Val Leu Thr Gln Asn Thr Ser Phe Pro Pro
        435                 440                 445

Gly Tyr Asn Ile Val Glu Val Asn Gly Ala Asp Gln Trp Ser Tyr Trp
    450                 455                 460

Leu Ile Glu Asn Asp Pro Gly Ala Pro Phe Thr Leu Pro His Pro Met
465                 470                 475                 480

His Leu His Gly His Asp Phe Tyr Val Leu Gly Arg Ser Pro Asp Glu
                485                 490                 495

Ser Pro Ala Ser Asn Glu Arg His Val Phe Asp Pro Ala Arg Asp Ala
            500                 505                 510

Gly Leu Leu Ser Gly Ala Asn Pro Val Arg Arg Asp Val Ser Met Leu
        515                 520                 525

Pro Ala Phe Gly Trp Val Val Leu Ser Phe Arg Ala Asp Asn Pro Gly
    530                 535                 540

Ala Trp Leu Phe His Cys His Ile Ala Trp His Val Ser Gly Gly Leu
545                 550                 555                 560

Gly Val Val Tyr Leu Glu Arg Ala Asp Asp Leu Arg Gly Ala Val Ser
                565                 570                 575

Asp Ala Asp Ala Asp Asp Leu Asp Arg Leu Cys Ala Asp Trp Arg Arg
            580                 585                 590

Tyr Trp Pro Thr Asn Pro Tyr Pro Lys Ser Asp Ser Gly Leu Lys His
        595                 600                 605

Arg Trp Val Glu Glu Gly Glu Trp Leu Val Lys Ala
    610                 615                 620
```

What we claim is:

1. A DNA construct containing a sequence encoding a *Myceliophthora* laccase.

2. The construct of Claim 1 which comprises a sequence encoding a *Myceliophthora thermophila* laccase.

3. The construct of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 2.

4. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 1.

5. The construct of Claim 1, which comprises the nucleic acid sequence contained in NRRL B-21261.

6. A substantially pure *Myceliophthora* laccase enzyme.

7. The enzyme of Claim 6 which is a *Myceliophthora thermophila* laccase.

8. The enzyme of Claim 6 which comprises the amino acid sequence depicted in SEQ ID NO. 2, or a sequence with at least about 80% homology thereto.

9. A recombinant vector comprising an DNA construct containing a sequence encoding a *Myceliophthora* laccase.

10. The vector of Claim 9 in which the construct is operably linked to a promoter sequence.

11. The vector of Claim 10 in which the promoter is a fungal or yeast promoter.

12. The vector of Claim 11 in which the promoter is the TAKA amylase promoter of *Aspergillus oryzae*.

13. The vector of Claim 11 in which the promoter is the glucoamylase (gluA) promoter of *Aspergillus niger* or *Aspergillus awamori*.

14. The vector of Claim 9 which also comprises a selectable marker.

15. The vector of Claim 14 in which the selectable marker is selected from the group consisting of *amdS*, *pyrG*, *argB*, *niaD*, *sC*, and *hygB*.

16. The vector of Claim 14 in which the selectable marker is the amdS marker of *Aspergillus nidulans* or *Aspergillus oryzae*, or the pyrG marker of *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus awamori*, or *Aspergillus oryzae*.

17. The vector of Claim 14 which comprises both the TAKA amylase promoter of *Aspergillus oryzae* and the amdS or pyrG marker of *Aspergillus nidulans* or *Aspergillus oryzae*.

18. A recombinant host cell comprising a heterologous nucleic acid construct containing a nucleic acid sequence encoding a *Myceliophthora* laccase.

19. The cell of Claim 18 which is a fungal cell.

20. The cell of Claim 19 which is an *Aspergillus* cell.

21. The cell of Claim 18 in which the construct is integrated into the host cell genome.

22. The cell of Claim 18 in which the construct is contained on a vector.

23. The cell of Claim 18 which comprises a construct containing a sequence encoding the amino acid sequence depicted in SEQ ID NO. 2..

24. A method for obtaining a laccase enzyme which comprises culturing a recombinant host cell comprising a DNA construct containing a nucleic acid sequence encoding a *Myceliophthora* laccase enzyme, under conditions conducive to expression of the enzyme, and recovering the enzyme from the culture.

25. A *Myceliophthora* enzyme obtained by the method of Claim 24.

26. An ascomycete or deuteromycete laccase having a specific activity of at least 30 SOU/mg on syringaldazine at optimum pH.

27. A method of enhancing yield of active recombinant copper-containing enzyme which comprises culturing a recombinant host cell comprising a DNA construct containing a sequence encoding a copper containing enzyme, under conditions conducive to expression of the enzyme, in the presence of at least about 0.02mM copper.

28. A method for polymerizing a lignin or lignosulfate substrate in solution which comprises contacting the substrate with a *Myceliophthora* laccase.

29. A method for in situ depolymerization in Kraft pulp which comprises contacting the pulp with a *Myceliophthora* laccase.

30. A method for oxidizing dyes or dye precursors which comprises contacting the dye with a *Myceliophthora* laccase.

31. A method for dyeing hair which comprises contacting a *Myceliophthora* laccase, in the presence or absence of at least one modifier, with at least one dye precursor, for a time and under conditions sufficient to permit oxidation of the dye precursor to a dye.

32. The method of claim 31 in which the dye precursor is selected from the group consisting of a diamine, aminophenol, and a phenol.

33. The method of claim 31, wherein the modifier, when used, is a meta-diamine, a meta-aminophenol or a polyphenol.

34. The method of claim 32 in which the dye precursor is a primary intermediate selected from the group consisting of an ortho- or para-diamine or aminophenol.

35. The method of claim 31 in which more than one dye precursor is used.

36. The method of claim 31 in which more than one modifier is used.

37. The method of claim 31 in which both a primary intermediate and a modifier are used.

38. A dye composition comprising a *Myceliophthora* laccase combined with at least one dye precursor.

39. A dye composition comprising a *Myceliophthora* laccase combined with at least one primary intermediate and at least one modifier.

40. A container containing a dye composition comprising a *Myceliophthora* laccase and at least one dye precursor in an oxygen-free atmosphere.

41. The container of claim 40 which contains at least one primary intermediate dye precusor combined with at least one modifier.

42. A method of polymerizing or oxidizing a phenolic or aniline compound which comprises contacting the phenolic or aniline compound with a *Myceliophthora* laccase.

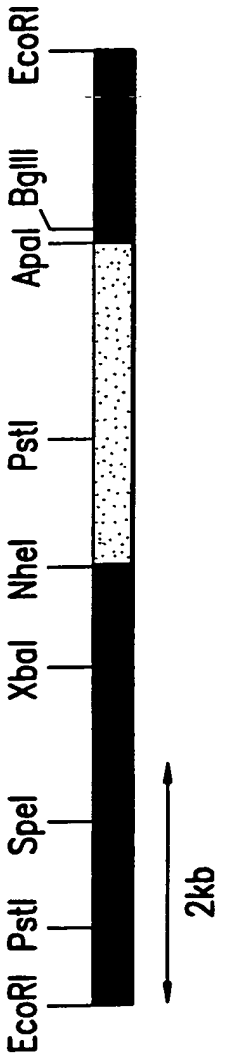

FIG. 1

```
gctagcttctttggtcaccgtcgttttcgcccgccccctccctccttcaaccccctgagtagtcggctaagcgatcctca  80

atctggtcttgtgaggtcacgtcctccagcagatgacagttcatcgagcgagtgatctccaccacccagaagggaggggg  160

gatgcgcgcatgctccaacataccctggtgtcgctagagacgtcgcggcatcagccttttcatcacaccgagcacgtcca  240

cggaccggctcctttcacccccgcgtcctccggaggattgagtcacgatatttcgggatgtgggaaggggggagagaaagg  320

agggggggaggggcggaaacatgttggatacgagctgcgcccctttccaacatcgagaacaggaagtcgttggtgtcggc  400

cgtaatgtctataaaacgaggctccttctcgtcgtcgacttgtctcaggttctctctctcgtccacaccaagccagtctt  480

gcctgagccacctgagccaccttcaactcatcatcttcagtcaagtcgttcattgacattgtgtctctctttctatcgag  560

tcggcttcccggcccttcaccacaacATGAAGTCCTTCATCAGCGCCGCGACGCTTTTGGTGGGCATTCTCACCCCTAGC  640
                                MetLysSerPheIleSerAlaAlaThrLeuLeuValGlyIleLeuThrProSer  -30

GTTGCTGCTGCCCCTCCATCCACCCCTGAGCAGCGCGACCTGCTCGTCCCGATCACGGAGAGGGAGGAGGCAGCCGTGAA  720
ValAlaAlaAlaProProSerThrProGluGlnArgAspLeuLeuValProIleThrGluArgGluGluAlaAlaValLy  -3

GGCTCGCCAGCAGAGCTGCAACACCCCCAGCAACCGGGCGTGCTGGACTGACGGATACGACATCAACACCGACTACGAAG  800
sAlaArgGlnGlnSerCysAsnThrProSerAsnArgAlaCysTrpThrAspGlyTyrAspIleAsnThrAspTyrGluV  25

TGGACAGCCCCGGACACGGGTGTTGTTCGGCCGgtgagtgctctcgttaattacgcttcggcgagttgcgcagatatatta  880
alAspSerProAspThrGlyValValArgPro                                                    35

aatactgcaaacctaagcaggagctgacatgcgacagTACACTCTGACTCTCACCGAAGTCGACAACTGGACCGGACCTG  960
                                TyrThrLeuThrLeuThrGluValAspAsnTrpThrGlyProA  50

ATGGCGTCGTCAAGGAGAAGGTCATGCTGGTTAACAgtacggcacccctttttcttgtcctaggatctgggtgatgtgcgt  1040
spGlyValValLysGluLysValMetLeuValAsnA                                              62

cgttgcccctgagagagactgaccgagcctttggctgcagATAGTATAATCGgtaattaattataccgccctgcctccag  1120
                                snSerIleIleG                                        66

cagccccagcagctcgagaagggtatctgaagttagtcaggcctgctgacctgaccggggccaacccaccatagGACCAA  1200
                                                                    lyProT          68

CAATCTTTGCGGACTGGGGCGACACGATCCAGGTAACGGTCATCAACAACCTCGAGACCAACGGgtatgtctgctgcttg  1280
hrIlePheAlaAspTrpGlyAspThrIleGlnValThrValIleAsnAsnLeuGluThrAsnGl                  89
```

FIG.2A

```
ctctcttgctctcctcgtccgcgactaataataatatcaactcgtgtggaaaacagCACGTCGATCCACTGGCACGGACT 1360
                                                        yThrSerIleHisTrpHisGlyLe  97

GCACCAGAAGGGCACCAACCTGCACGACGGCGCCAACGGTATCACCGAGTGCCCCGATCCCCCCCCAAGGGAGGGAGGAAGG 1440
uHisGlnLysGlyThrAsnLeuHisAspGlyAlaAsnGlyIleThrGluCysProIleProProLysGlyGlyArgLysV  124

TGTACCGGTTCAAGGCTCAGCAGTACGGGACGAGCTGGTACCACTCGCACTTCTCGGCCCAGTACGGCAACGGCGTGGTC 1520
alTyrArgPheLysAlaGlnGlnTyrGlyThrSerTrpTyrHisSerHisPheSerAlaGlnTyrGlyAsnGlyValVal  150

GGGGCCATTCAGATCAACGGACCGGCCTCGCTGCCGTACGACACCGACCTGGGTGTGTTCCCCATCAGCGACTACTACTA 1600
GlyAlaIleGlnIleAsnGlyProAlaSerLeuProTyrAspThrAspLeuGlyValPheProIleSerAspTyrTyrTy  177

CAGCTCGGCCGACGAGCTGGTGGAACTCACCAAGAACTCGGGCGCGCCCTTCAGCGACAACGTCCTGTTCAACGGCACGG 1680
rSerSerAlaAspGluLeuValGluLeuThrLysAsnSerGlyAlaProPheSerAspAsnValLeuPheAsnGlyThrA  204

CCAAGCACCCCGAGACGGGCGAGGGCGAGTACGCCAACGTGACGCTCACCCCGGGCCGGCGGCACCGCCTGCGCCTGATC 1760
laLysHisProGluThrGlyGluGlyGluTyrAlaAsnValThrLeuThrproGlyArgArgHisArgLeuArgLeuIle  230

AACACGTCGGTCGAGAACCACTTCCAGGTCTCGCTCGTCAACCACACCATGTGCATCATCGCCGCCGACATGGTGCCCGT 1840
AsnThrSerValGluAsnHisPheGlnValSerLeuValAsnHisThrMetCysIleIleAlaAlaAspMetValProVa  257

CAACGCCATGACGGTCGACAGCCTCTTCCTCGGCGTCGGCCAGCGTTACGATGTCGTCATCGAAGCCAACCGAACGCCCG 1920
lAsnAlaMetThrValAspSerLeuPheLeuGlyValGlyGlnArgTyrAspValValIleGluAlaAsnArgThrProG  284

GGAACTACTGGTTTAACGTCACATTTGGCGGCGGCCTGCTCTGCGGCGGCTCCAGGAATCCCTACCCGGCCGCCATCTTC 2000
lyAsnTyrTrpPheAsnValThrPheGlyGlyGlyLeuLeuCysGlyGlySerArgAsnProTyrProAlaAlaIlePhe  310

CACTACGCCGGCGCCCCCCGGCGGCCCGCCCACGGACGAGGGCAAGGCCCCCGGTCGACCACAACTGCCTGGACCTCCCCAA 2080
HisTyrAlaGlyAlaProGlyGlyProProThrAspGluGlyLysAlaProValAspHisAsnCysLeuAspLeuProAs  337

CCTCAAGCCCCGTCGTGGCCCCGCGACGTGCCCCTGAGCGGCTTCGCCAAGCGGGCCGACAACACGCTCGACGTCACCCTCG 2160
nLeuLysProValValAlaArgAspValProLeuSerGlyPheAlaLysArgAlaAspAsnThrLeuAspValThrLeuA  364

ACACCACGGGCACGCCCCTGTTCGTCTGGAAGGTCAACGGCAGCGCCATCAACATCGACTGGGGGAGGGCCGTCGTCGAC 2240
spThrThrGlyThrProLeuPheValTrpLysValAsnGlySerAlaIleAsnIleAspTrpGlyArgAlaValValAsp  390

TACGTCCTCACGCAGAACACCAGCTTCCCACCCGGGTACAACATTGTCGAGGTGAACGGAGCTGATCAGgtaagaaaaag 2320
TyrValLeuThrGlnAsnThrSerPheProProGlyTyrAsnIleValGluValAsnGlyAlaAspGln            413

gggaccgcaggggtgctgctgcaagtacaccttgctcgccctcctgttcttccttaataactacctcccaaccctccccc 2400

ctaattaattcactttaaaggccgatcaagactgaccgagccccctctctttgcagTGGTCGTACTGGTTGATCGAGAAC 2480
                                                        TrpSerTyrTrpLeuIleGluAsn  421
```

FIG.2B

```
GATCCCGGCGCACCTTTCACCCTACCGCATCCGATGCACCTGCACgtaagttggatacatatatatatatatatacat  2560
AspProGlyAlaProPheThrLeuProHisProMetHisLeuHis                                    436

tgctttcctggctcgctcccttaaataaaattaaataaccaaaaataacaaaaaaaaagGGCCACGACTTTTACGTGCTGG  2640
                                                              HlyHisAspPheTyrValLeuG  444

GCCGCTCGCCCCGACGAGTCGCCGGCATCCAACGAGCGGCACGTGTTCGATCCGGCGCGGGACGCGGGCCTGCTGAGCGGG  2720
lyArgSerProAspGluSerProAlaSerAsnGluArgHisValPheAspProAlaArgAspAlaGlyLeuLeuSerGly  470

GCCAACCCTGTGCCGGCGGGACGTGTCGATGCTGCCGGCGTTCGGGTGGGTGGTGCTGTCCTTCCGGGCCGACAACCCGGG  2800
AlaAsnProValArgArgAspValSerMetLeuProAlaPheGlyTrpValValLeuSerPheArgAlaAspAsnProGl  497

CGCCTGGCTGTTCCACTGCCACATCGCCTGGCACGTCTCGGGCGGCCTGGGCGTCGTCTACCTCGAGCGCGCCGACGACC  2880
yAlaTrpLeuPheHisCysHisIleAlaTrpHisValSerGlyGlyLeuGlyValValTyrLeuGluArgAlaAspAspL  524

TGCGCGGGGCCGTCTCGGACGCCGACGCCGACGACCTCGACCGCCTCTGCGCCGACTGGCGCCGCTACTGGCCTACCAAC  2960
euArgGlyAlaValSerAspAlaAspAlaAspAspLeuAspArgLeuCysAlaAspTrpArgArgTyrTrpProThrAsn  550

CCCTACCCCAAGTCCGACTCGGGCCTCAAACACCGCTGGGTCGAGGAGGGCGAGTGGCTGGTCAAGGCGtgagcgaagga  3040
ProTyrProLysSerAspSerGlyLeuLysHisArgTrpValGluGluGlyGluTrpLeuValLysAla***        573

ggaaaaaggaaacaaagaggggggggggctagttcctatttttgctttttttttttgttcttgtccttgtgctggcgg  3120

ttccctggtaaaggagaaggggcccccaagttcgagtgggtgtgtgatcgggtaaatattatcaagagatct  3192
```

FIG.2C

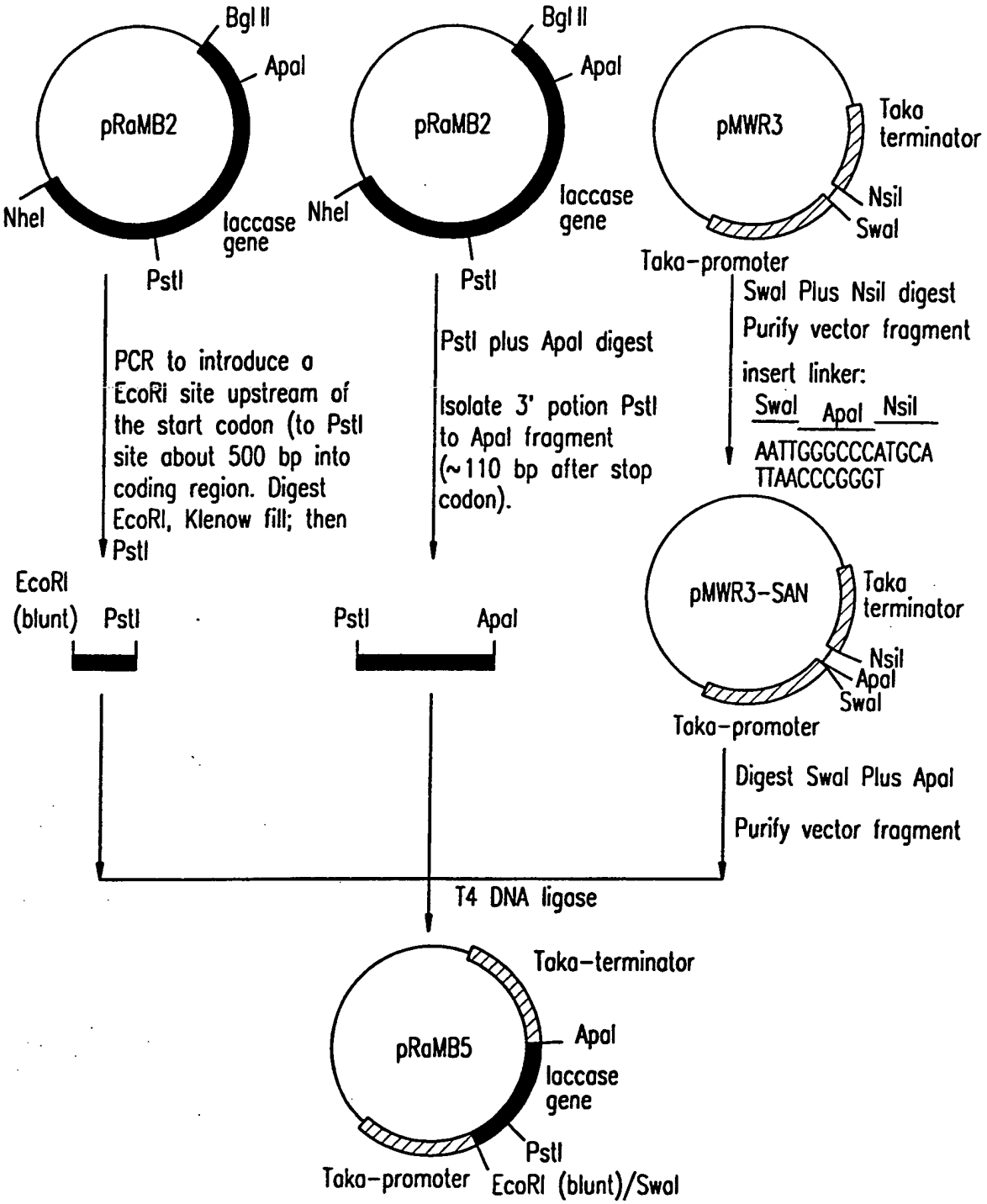
Bgl II
ApaI
pRaMB2
NheI
laccase gene
PstI
PCR to introduce a EcoRI site upstream of the start codon (to PstI site about 500 bp into coding region. Digest EcoRI, Klenow fill; then PstI
EcoRI (blunt) PstI
PstI plus ApaI digest
Isolate 3' potion PstI to ApaI fragment (~110 bp after stop codon).
PstI ApaI
pMWR3
Taka terminator
NsiI
SwaI
Taka-promoter
SwaI Plus NsiI digest
Purify vector fragment
insert linker:
SwaI ApaI NsiI
AATTGGGCCCATGCA
TTAACCCGGGT
pMWR3-SAN
Taka terminator
NsiI
ApaI
SwaI
Taka-promoter
Digest SwaI Plus ApaI
Purify vector fragment
T4 DNA ligase
Taka-terminator
pRaMB5
ApaI
laccase gene
PstI
Taka-promoter
EcoRI (blunt)/SwaI

FIG.3

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N1/15 A61K7/13 A61K7/06 D21C5/00
//(C12N1/15,C12R1:66)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K D21C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,O, X	ABSTRACTS OF PAPERS, vol.209, no.1-2, April 1995, ANAHEIM, CA BERKA R. ET AL. 'Cloning of laccases from the thermophilic fungi Myceliophthora thermophila and Scytalidium thermophilum and their heterologous expression in Aspergillus oryzae' see BIOT 196 ---	1,2,6,7, 9,10, 18-20, 24-26
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.263, no.2, 1988, BALTIMORE, MD US pages 885 - 896 GERMANN U. ET AL. 'Characterization of two allelic forms of Neurospora crassa laccase' see page 885, right column --- -/--	26

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 August 1995	11.09.95

Name and mailing address of the ISA	Authorized officer
European Patent ffice, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Espen, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 317 243 (KYOWA MEDEX CO. LTD.) 24 May 1989	
P,X	WO,A,95 01426 (NOVO NORDISK A/S) 12 January 1995 see page 19; claim 22	6,7,25, 26

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0317243	24-05-89	JP-A-	1128797	22-05-89
		DE-D-	3851304	06-10-94
		DE-T-	3851304	26-01-95
		US-A-	5196312	23-03-93
WO-A-9501426	12-01-95	AU-B-	6924594	24-01-95